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(54) Title: RECOMBINANT MOLECULES WITH REDUCED IMMUNOGENICITY, METHODS AND INTERMEDIATES FOR OBTAINING THEM AND THEIR USE IN PHARMACEUTICAL COMPOSITIONS AND DIAGNOSTIC TOOLS

Amino acid residues	T cell clone	
2140 2150 2160	B3:6	D9:E9
IFNPPIIARYIRLHPT	-	-
IIARYIRLHPTHYSIRST	++	++
ARYIRLHPTHYSIRST	+	+
YIRLHPTHYSIRST	+	-
RLHPTHYSIRST	-	-
IIARYIRLHPTHYSI	+	+
IIARYIRLHPTHY	-	-

(57) Abstract: The invention provides a recombinant mammalian protein modified to eliminate or reduce by at least about 80 % the activation of at least one T-cell clone derived from a mammal with antibody against the wild-type protein, with respect to activation by the wild-type protein, the said recombinant protein having at specific activity higher than 0.1. The recombinant protein is useful for making a pharmaceutical composition for the prevention or treatment of a disease induced by a lack or a dysfunction of a human protein such as hemophilia.

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RECOMBINANT MOLECULES WITH REDUCED IMMUNOGENICITY, METHODS AND INTERMEDIATES FOR OBTAINING THEM AND THEIR USE IN PHARMACEUTICAL COMPOSITIONS AND DIAGNOSTIC TOOLS.

The present invention relates to recombinant molecules with reduced immunogenicity, methods and intermediates for producing them and therapeutic and diagnostic applications resulting therefrom. More particularly, this invention relates to recombinant mammalian proteins, e.g. coagulation factors, with reduced immunogenicity as active ingredients for the treatment of patients with diseases induced by a dysfunction of a protein, in particular various types of hemophilia.

BACKGROUND OF THE INVENTION

Factor VIII (hereinafter referred as FVIII) is a protein providing important coagulant cofactor activity and is one of human clotting factors with a rather high molecular weight (about 265,000) and a very low normal plasma concentration (0.0007 µmole/litre). With its 2,332 amino-acid residues, FVIII is one of the longest known polypeptide chains and is synthesized in the liver, the spleen and the placenta. Its gene has been shown to include 186,000 nucleotides. FVIII circulates as inactive plasma protein. Factors V and VIII are homologous proteins sharing a common structural configuration of triplicated A domains and duplicated C domains with structurally divergent B domains connecting the A2 and A3 domains.

The human FVIII gene was isolated and expressed in mammalian cells, as reported by various authors, including Wood et al. in *Nature* (1984) 312:330-337 and the amino-acid sequence was deduced from cDNA. U.S.Patent No. 4,965,199 discloses a recombinant DNA method for producing FVIII in mammalian host cells and purification of human FVIII. The human FVIII detailed structure has been extensively investigated. The cDNA nucleotide sequence encoding human FVIII and predicted amino-acid sequence have been disclosed for instance in U.S.Patent No. 5,663,060. In a FVIII molecule, a domain may be defined as a continuous sequence of amino-acids that are defined by internal amino-acid sequence homology and sites of proteolytic cleavage by thrombin. Unless

otherwise specified, FVIII domains include the following amino-acid residues, when the sequences are aligned with the human amino-acid sequence: A1, residues 1-372; A2, residues 373-740; B, residues 741-1648; A3, residues 1690-2019; C1, residues 2020-2172; C2, residues 2173-2332. The remaining sequence, residues 1649-1689, is usually referred to as the FVIII light chain activation peptide. FVIII is produced as a single polypeptide chain which is rapidly cleaved after secretion to form a heterodimer made of a heavy chain constituted by A1-A2 domains and variable lengths of the B domain, and a light chain made of the A3-C1-C2 domains. The two chains are non-covalently bound by divalent cations. Human recombinant FVIII may be produced by genetic recombination in mammalian cells such as CHO (Chinese Hamster Ovary) cells, BHK (Baby Hamster Kidney) cells or other equivalent cells.

Pratt et al. in *Nature* (1999) 402:439-42 disclose the detailed structure of the carboxy-terminal C2 domain of human FVIII, which contains sites that are essential for its binding to von Willebrand factor and to negatively charged phospholipid surfaces. This structure, which reveals a beta-sandwich core from which two beta-turns and a loop display a group of solvent-exposed hydrophobic residues, partly explains mutations in the C2 region that lead to bleeding disorders in hemophilia A. According to Gale et al. in *Thromb. Haemost.* (2000) 83:78-85, of the at least 250 missense mutations that cause FVIII deficiency and hemophilia A, 34 are in the C domains.

Haemophilia A is a disease characterized by insufficient quantity of functionally active FVIII molecules. The disease, which affects 1/10,000 males, is classified according to the level of functional FVIII in severe (< than 1% FVIII), mild (1-5%) and moderate forms (>5%).

FVIII is a cofactor of the intrinsic pathway of the coagulation cascade, which acts by increasing the proteolytic activity of activated factor IX over factor X, in the so-called tenase complex formation. Patients suffering from hemophilia A present with bleedings which are either spontaneous in the severe form of the disease, or occur after trauma in the mild/moderate forms.

Hemophilia A patients are usually treated by replacement therapy, which consists in infusing human FVIII either purified from pools of donor plasma, or obtained by cDNA recombination technology. For instance, U.S.Patents No. 5,618,788 and No. 5,633,150 disclose producing functional species of human FVIII via recombinant DNA technology. In particular, they disclose (i) isolated DNA comprising a sequence encoding functional human FVIII, and (ii) a replicable expression vector capable, in a transfectant culture of cells, of expressing the said DNA sequence. They also express the idea that various human FVIII derivatives may potentially exist and be prepared by single or multiple amino acid deletions, substitutions, insertions or inversions, e.g. by means of site directed mutagenesis of the underlying DNA. These documents however do not disclose what these deletions, substitutions, insertions or inversions should be in order to have specific usefulness in diagnostic or therapeutic applications.

U.S.Patents No. 5,364,771 and No. 5,663,060 disclose purified hybrid factor VIII molecules comprising non-human mammalian and human amino acid sequences and having procoagulant activity *in vitro*. They also disclose compositions comprising such molecules combined with a pharmaceutically acceptable carrier, which are useful in treating human patients having antibodies to FVIII that inhibit coagulation activity. However these hybrid FVIII molecules suffer from the well known disadvantages of heterologous systems, i.e. although for instance porcine FVIII can be administered to humans with inhibitor antibodies, because there is only limited cross-reactivity between human and porcine FVIII and porcine FVIII is not inactivated by patient inhibitor antibodies, however tolerance to porcine FVIII is only transient and high titer antibodies towards both human and porcine FVIII can be detected in plasma of such patients after only a few days of administration. This indicates that the repertoire of B lymphocytes contains cells able to produce antibodies recognizing porcine FVIII.

One of the major complications of the replacement therapy is the elicitation of an immune response towards the infused compound. Thus, antibodies to FVIII

preclude further infusion, as exogenous FVIII is immediately neutralized by circulating anti-FVIII antibodies.

Different factors are known to be associated with the emergence of anti-FVIII antibodies, also called inhibitors. Thus, patients with the severe form of the disease are more prone to develop inhibitors than patients with the mild/moderate form. Some FVIII products have also been associated with a higher incidence of inhibitors. Altogether, about 25% of haemophilia A patients produce inhibitory anti-FVIII antibodies.

Factor IX (hereinafter referred as FIX) is a globular protein which has a molecular weight of about 70,000 daltons and which, in a normal individual, is constantly produced in the liver and circulates at a normal blood plasma concentration of about 5 µg/ml. FIX is a vitamin K-dependent protein which also participates in blood coagulation. It is synthesized in the form of a zymogen and undergoes three types of post-translational modifications before being secreted into the blood: (i) vitamin-K-dependent conversion of glutamic acid to carboxyglutamic acid, (ii) addition of hydrocarbon chains and (iii) beta-hydroxylation of an aspartic acid. It participates in the blood coagulation cascade and is used for the treatment of hemophilia B patients

Although FIX inhibitors are far less common and occur in about 2 to 3% of boys with hemophilia B (compared to an occurrence of about 30 to 50% of inhibitors in boys with hemophilia A), approximately half of such cases where inhibitors against FIX occur are accompanied by the occurrence of anaphylaxis or severe hypersensitivity reactions to any FIX-containing product, according to Lusher in *Best Pract. Res. Clin. Haematol.* (2000) 13:457-468.

Anti-FVIII antibodies are mostly immunoglobulin G (IgG) antibodies. The production of such antibodies is believed to be "T-cell dependent", which means that help provided by specific T lymphocytes is required for efficient activation of B lymphocytes.

An immune response towards soluble antigens, such as FVIII, requires that the antigen is first processed by specialized cells, called antigen-presenting cells.

The function of such cells is precisely to present the antigen after processing to specific T cells. The latter can in turn help B lymphocytes to mature and secrete antibodies, i.e. specific T cell activation is a necessary step occurring before B cell activation. Therefore, preventing the activation of FVIII-specific T cells would also prevent B cell activation and thereby antibody production.

T cells recognize small stretches of amino acids presented in the context of class II major histocompatibility complex molecules (hereinafter referred as MHC-class II). In addition, T cell epitopes are organized according to a hierarchy consisting of immuno-dominant or major epitopes, minor epitopes and cryptic epitopes. Major epitopes are recognized by a majority of individuals and represent the epitopes first recognized in the elicitation of an immune response. Eliminating such major T cell epitopes could therefore be sufficient to prevent T cell activation towards FVIII. Altogether, it is likely that only a limited number of T cells epitopes would need to be removed from the FVIII molecule in order to prevent the formation of inhibitory antibodies.

There are two methods by which a T cell epitope can be eliminated. This can first be achieved by the substitution of some amino-acids involved in the anchoring of the peptide containing the T cell epitope in major histocompatibility complexes at the surface of antigen-presenting cells. This first method results in a lack of peptide presentation, which is independent of the specificity of the T cell receptor. The second method consists in altering the amino-acid residues that are directly involved in recognition by the T cell receptor.

Therefore there is a general need in the art for a method suitable to reduce the immune response of a protein, and more specifically to identify the T cell epitopes of proteins, in particular the T cell epitopes of blood coagulation factors such as the FVIII molecule and factor IX, which are associated with the activation of T cells participating in the production of inhibitory antibodies. There is also a need in the art for modified proteins that are able to substantially reduce the activation of T cells while keeping a high specific activity in replacement therapy. Apart from blood coagulation factors, a similar concern has been expressed for the

enzyme replacement therapy developed for mucopolysaccharidose patients, since immune responses have been reported in animal models and in human Gaucher patients. There is also a broad concern in the art that the development of antibodies to replaced proteins may limit the success of many human gene therapy approaches. It is the purpose of the present invention to address these various needs.

SUMMARY OF THE INVENTION

A first aim of the present invention is a method to identify the T cell epitopes of proteins, in particular blood coagulation factors such as the FVIII molecule, which are associated with activation of T cells participating in the production of protein-specific antibodies, e.g. inhibitory antibodies. After effective performance of this identification method, amino-acids of such epitopes may then be substituted or deleted in order to prevent T cell recognition. Amino-acid residues that are directly involved in the anchoring into MHC-class II determinants are preferably first identified and then substituted or deleted in order to prevent anchoring and thereby prevent T cell activation. Alternative possibilities will also be considered, which target amino-acid residues recognized by the T cell antigen receptor or flanking residues involved in the affinity of T cell recognition. Residue substitutions and/or deletions which are able to preserve the major part of the protein biological activity, e.g. essentially all of the FVIII pro-coagulant function, are then selected.

The invention thus provides a recombinant protein modified to eliminate or reduce by at least about 80% the activation of at least one T-cell clone derived from a mammal with antibody against the wild-type mammalian protein, with respect to activation by the wild-type mammalian protein, the said recombinant protein having a specific activity higher than about 0.1. Preferably, the T cell clone is derived from a human. More preferably, the said protein is a coagulation factor such as FVIII or FIX. More preferably, the recombinant protein is in a purified form.

The invention also provides a method to generate a mammalian protein-specific T-cell clonal cell line by using a cell line expressing protein-specific antibodies, or fragments thereof, on its surface. Preferably the said cell line is a lymphoblastoid

cell line. For instance, the invention provides T-cell clonal cell lines obtained by using as antigen-presenting cell the cell line KRIX 1 deposited with the Belgian Coordinated Collections of Micro-organisms under accession number LMBP 5089CB. Preferably, the protein involved in this method is a coagulation factor such as FVIII or FIX. More preferably, cell lines expressing the protein-specific antibodies are obtained by transfection or transduction with an expression vector for the protein-specific antibody or fragment thereof. More preferably the method comprises, in the following order, at least one of the following steps :

- identifying a peptide which has an epitope recognized by the clonal T-cell line by using a synthetic peptide library corresponding to the said protein,
- using the T cell clonal cell line to identify modifications of the T-cell epitope eliminating or reducing by at least about 80% its ability to activate the T-cell clonal cell line,
- producing a recombinant protein carrying a modification identified in the previous identification step,
- using the T-cell clone to verify that the modified protein does not provoke more than 20% T-cell activation by comparison to the wild-type protein, and
- controlling the activity of the modified protein by means of a suitable protein functional assay.

The present invention further encompasses a recombinant protein obtainable by this method, for instance a recombinant protein carrying, in respect of the wild-type protein, a substitution of a single residue and/or one amino-acid deletion. As a typical example thereof, this invention considers a recombinant human FVIII protein carrying a substitution by another residue in the region between residues 2144 and 2161, for instance a substitution at residue 2153. This invention also relates to a recombinant protein carrying in respect of the wild-type protein several modifications located in one domain or combination of domains of the protein.

In another embodiment, this invention provides a peptide identified during the first step of the method as previously disclosed. Such a peptide preferably encompasses residues which can be mutated or deleted to eliminate or reduce by

at least about 80% the activation of at least one T-cell clone activated by a wild-type protein. For instance, when the protein is a coagulation factor such as the human FVIII, the said peptide includes at least residues 2144 to 2161 thereof.

This invention also provides *in vitro* use of such a peptide for evaluating T-cell reactivity, and *in vivo* use of such a peptide for evaluating and/or modulating T-cell reactivity.

In still another embodiment, this invention provides a DNA sequence coding for a recombinant protein such as previously defined, in particular the DNA sequence of human FVIII carrying one single mutation at residue 2153. The invention further relates to an expression vector including such a DNA sequence and a suitable promoter.

This invention also relates to a pharmaceutical composition comprising an effective amount of a recombinant protein, a peptide or an expression vector such as previously disclosed, and a pharmaceutically acceptable carrier. Finally, the invention provides a method for the prevention or treatment of a disease induced by a lack or a dysfunction of a human protein, comprising administering to a patient in need thereof an effective amount of a recombinant protein, a peptide or an expression vector such as previously disclosed. The said protein may be a coagulation factor such as FVIII (the disease to be treated being hemophilia A) or FIX (the disease to be treated being hemophilia B).

Importantly, the present invention is widely applicable to any mutant or variant of the relevant protein, in particular to any mutant or variant of the FVIII or FIX molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows IFN-γ production following activation of T-cell clonal cell lines with human factor VIII.

Figure 2 shows stimulation of T-cell clonal cell lines with recombinant human factor VIII fragments.

Figure 3 shows the results of T-cell clone epitope mapping with synthetic peptides of the C1 domain of human factor VIII.

Figure 4 shows that non-specific B cell lines present human FVIII peptides but not intact FVIII.

Figure 5 shows that point mutations alter the T-cell epitope recognized by clonal cell lines.

DEFINITIONS

As used herein, "modified protein" denotes any recombinant protein molecules in which a single or a small number of amino-acids have been either substituted by any other amino-acid residue or deleted. Such amino-acid substitution or deletion can be located anywhere in the protein molecule. It also denotes protein molecules in which amino-acid residues have been substituted and/or deleted at more than a single location. In the latter case, any combination of substitution and deletion can be considered.

As used herein, the terms "epitope", "antigenic site" and "antigenic determinant" are used synonymously and are defined as a portion of the protein that is specifically recognized by an antibody or by the antigen receptor of a T cell. It can consist of any number of amino-acid residues and can be dependent upon the primary, secondary or tertiary structure of the protein. Thus, a protein that includes at least one epitope may be used as a reagent in the diagnostic assays.

As used herein, a "T cell epitope" is a stretch of amino-acids from the sequence of a protein and which varies in length between about 7 and 30 amino-acids. This epitope contains (i) residues enabling it to anchor into MHC class II molecules and (ii) distinct residues that are recognized by the antigen receptor of the corresponding T cell.

As used herein, a "B cell epitope" is a set of amino-acids that are present either as a sequence of amino-acids from a protein molecule, or as discontinuous residues located at a distance from each other but brought together in the 3-D structure of the molecule. The B cell epitope is recognized by antibodies at the surface of specific B cells and by soluble antibodies produced by such B cells.

As used herein, "specific activity" makes reference to the ratio of the biological activity of a protein in a certain diagnostic assay to the biological activity

of the corresponding wild-type protein in the same assay. For instance, as applied to FVIII, it refers to the activity that will correct the coagulation defect of human FVIII deficient plasma and is measured in units of clotting activity per milligram total FVIII protein in an assay in which the clotting time of human FVIII deficient plasma is compared to that of normal human plasma. In this assay, the shorter the time for clot formation, the greater the activity of the FVIII being assayed.

As used herein, "expression vector" means a vector which is capable of expressing a DNA sequence contained therein, where such sequence is operably linked to another sequence, such as a promoter, capable of effecting their expression. The expression vector replicates in the host cell, either by means of an intact operable origin of replication or by functional integration into the cell chromosome. In general, an expression vector useful in recombinant DNA technology is in the form of a "plasmid" which refers to circular double stranded DNA loops. However the invention includes all such other forms of expression vectors which serve equivalent functions.

DETAILED DESCRIPTION OF THE INVENTION

Although the present invention will be explained in details with respect to human FVIII, it is based on a concept widely applicable to mammalian proteins, in particular to blood coagulation factors, and therefore should not be interpreted solely in connection with human FVIII but should be extended to any protein of mammalian origin, which is known to be specifically involved in a specific disease. As briefly stated previously, the invention involves a method to produce a recombinant mammalian protein with reduced immunogeneicity, comprising the steps of :

(a) isolating from a mammal antibody-producing T-cells against a wild-type mammalian protein,

(b) identifying the major T-cell epitopes of the said wild-type mammalian protein which are associated with activation of T cells obtained in step (a), and

(c) substituting or deleting amino-acids of the major T-cell epitopes identified in step (b) in such way as to eliminate or reduce by at least about 80% T cell activation.

This method may further comprise the step of selecting the amino-acid substitutions or deletions of step (c) which are able to preserve a specific activity of the recombinant mammalian protein higher than about 0.1. Furthermore, the identifying step (b) may be performed by means of a peptide library from the wild-type mammalian protein.

More preferably, this invention is applicable to proteins which (i) are in current use or have been suggested for use in a therapeutic treatment and (ii) give rise to the occurrence of inhibitory antibodies in the patient. A non limiting list of such proteins includes:

- proteins involved in diseases of the immune system such as the gene product of the FMF gene responsible for familial mediterranean fever, the interleukin-2 receptor gamma (IL2RG) gene or adenosine deaminase (ADA) gene in severe combined immunodeficiency;
- proteins involved in metabolic disorders such as phenylalanine hydroxylase (PAH) in phenylketonuria, apolipoprotein E in Gauchers disease, ornithine ketoacid aminotransferase (OAT) in gyrate atrophy of the choroid coating retina, ABC1 (ATP-binding cassette) in Tangier disease , beta-hexosaminidase A (HEXA) in Tay-Sachs disease;
- proteins involved in diseases of the musculature such as the cytoskeletal protein dystrophin in Duchenne muscular distrophy, the survival motor neuron gene (SMN1) in spinal muscular atrophy (SMA);
- proteins involved in diseases of the nervous system such as the superoxide dismutase (SOD1) in amyotrophic lateral sclerosis (ALS), the gene product of the FMR1 gene in fragile X syndrome;
- proteins involved in diseases caused by a disfunction of cell signaling such as the gene product of the ATM gene in ataxia telangiectasia, the gene product

GLC1A gene in glaucoma, the gene product PAX3 in Waardenburg syndrome (WS), WRN helicase in Werner's disorder ;

- proteins involved in diseases caused by a disfunction of transporter proteins such as the gene product of the CFTR gene in cystic fibrosis, the copper tranporter protein encoded by the ATP7B gene in Wilson's disease.

The skilled person will readily be able to take benefit from the invention by first determining the probability to be successful in performing the invention for a given protein with minimal investigation, i.e. without undue burden and time efforts, according to the following instructions. First, the skilled person will generate at least a T-cell clonal cell line reacting against the protein of interest, using methods standard in the art. In this respect, a representative collection of T cell clones must be established in order to identify as many T cell epitopes as possible. Then, a peptide library of the protein of interest will be prepared, using peptide synthesis procedures common in the art. Then, the first step of the method of the invention, i.e. identifying by means of the peptide library corresponding to the protein of interest at least one peptide which has an epitope recognized by the clonal T-cell line, is performed. After this step, it becomes possible to identify the peptide that generates T cell clone activation. From this information, the skilled person is able to (i) derive the number of epitopes involved and (ii) evaluate where such epitopes are located on the protein of interest. If the number of epitopes involved is higher than about 20, it is likely that the invention will require extensive investigations which will make it less attractive. On the contrary, If the number of epitopes involved is not above about 20, then the invention will likely be helpful without an undue burden of effort and, therefore, the second step of the method of the invention, i.e. using the T cell clonal cell line to identify modifications of the T-cell epitope eliminating or reducing by at least about 80% its ability to activate the T-cell clonal cell line, will be performed. The skilled person readily understands that the amount of effort will depend not only upon the number of epitopes to be modified but also upon whether these are located in a region of the protein which is critical for its functional activity and/or its structural integrity.

Mild/moderate haemophilia A patients rarely produce inhibitory antibodies towards infused FVIII. This is due to the lower frequency of FVIII administration in such patients, who suffer from bleedings only after trauma or during surgery. However, this low frequency of inhibitors is also due to the fact that such patients having significant levels of circulating FVIII had the opportunity to become immunologically unresponsive against FVIII. The mechanisms by which such unresponsiveness develops are well known to those skilled in the art and can be found in recently published reviews such as *Immunological Tolerance* (1998) Novartis Foundation Symposium 215, Wiley, Chichester. Briefly, during the ontogeny of the immune system, lymphocytes capable to recognize and to be activated by self antigens are eliminated from the repertoire by deletion mechanisms occurring in the bone marrow (B cells) and in the thymus (T cells).

Patients with mild/moderate haemophilia A are therefore expected to have become tolerant towards all FVIII T cell epitopes, except those which are affected by the mutation/deletion responsible for the haemophilia phenotype. If an inhibitor antibody is formed, then the T cells required for antibody production should be located close to or at the said mutated/deleted site. In support of this, epidemiological studies in mild/moderate haemophilia A patients have identified a preferential association of inhibitory antibodies with mutations located within discrete regions of the FVIII molecule, such as parts of the C1 and of the C2 domains, as reported by Hay et al., *Thromb.Haemost.* (1998) 79: 762.

From peripheral blood lymphocytes of patients with inhibitor and mild/moderate haemophilia A, it is possible to expand the population of FVIII-specific T cells which are potentially involved in the production of antibodies. Such T cells can be cloned by methods well known in the art. However, we discovered that efficient characterization of such T cell lines required the use of FVIII-specific B cell lines as antigen-presenting cells.

The following is an example of a method to generate and identify cell lines expressing coagulation factor antibodies at their surface. Antigen-presenting cells activate CD4+ T cells that recognize peptides bound to MHC class II molecules.

For efficient activation, T cells require peptide presentation in the context of self MHC class II molecules. Therefore, cell lines expressing FVIII-specific surface immunoglobulins should express MHC class II molecules identical to those expressed by the individual from whom FVIII-specific T cell clones are to be derived. Such cell lines can be obtained by first immortalizing the patient's B lymphocytes with the Epstein-Barr virus, according to methods well known by those skilled in the art, for instance as disclosed by Jacquemin et al. in *Blood* (1998) 92:496. In a second step, FVIII-specific B cells are selected by screening cell culture supernatants for the presence of anti-FVIII antibodies. Antibodies towards FVIII are identified by incubating cell culture supernatants in microtitration polystyrene plates coated with FVIII. The binding of specific antibodies is detected by addition of an anti-human IgG reagent coupled to an enzyme. Addition of an enzyme substrate which is converted to a colored compound in the presence of the enzyme allows the detection of specific antibodies. Such method referred to as enzyme-linked immunoassay (ELISA) is well known to those skilled in the art and described in details e.g. in Current Protocols in Immunology, chapter 2, John Wiley & Sons (1994). Microcultures producing anti-FVIII antibodies are then expanded and cloned. Clonal cell lines are then further selected for the production of anti-FVIII antibodies, e.g. according to Jacquemin et al. (cited *supra*).

Alternatively, cell lines which do not produce anti-FVIII antibodies can be transformed in order to let them express FVIII-specific antibodies, provided that the selected cell lines express MHC class II molecules identical to the patients from whom T lymphocytes are taken for cloning. Such cell lines can be obtained by first immortalizing patient's B lymphocytes with the Epstein-Barr virus. In a second step, the cell lines are transformed with an expression vector coding for a FVIII-specific immunoglobulin. For this purpose, the complete cDNA encoding the heavy and light chains of a FVIII specific antibody is cloned using the cDNA obtained from a FVIII-specific cell line. Human lymphoblastoid cell lines (Jacquemin et al. cited *supra*) or mouse hybridomas (according to Gilles et al. in *Blood* (1993) 82:2452) producing anti-FVIII antibodies can be used as a source of such cDNA. The

introduction of the gene coding for the anti-FVIII antibody can then be carried out by transfection or transduction of the target cell. In order to prepare DNA for transfection, the complete cDNA encoding the heavy and light chains of the FVIII-specific antibody is cloned in a eukaryotic expression vector designed for the independent expression of two genes from a single plasmid, such as pBudCEA4.0 (available from Invitrogen, Groningen, The Netherlands). Importantly, the heavy chain must contain the transmembrane and intracytoplasmic portions of surface immunoglobulin. These portions can be cloned by polymerase chain reaction using cDNA of a lymphoblastoid cell line such as BO2C11 (Jacquemin et al. cited *supra*) by methods well known by those skilled in the art. CHO cells are then transfected with the expression vector. Microcultures containing cells producing anti-FVIII antibodies are then expanded and cloned. Clonal cell lines are then further selected for the production of anti-FVIII antibodies. Cell surface expression of the anti-FVIII antibody can be detected by labelling the cells with FVIII labelled with biotin or a fluorescent dye using techniques well known by those skilled in the art, as taught e.g. by Current Protocols in Immunology, Chapter 5, John Wiley & Sons, Inc. (1994).

Alternatively, the target cell can be transduced with a gene coding for a FVIII-specific antibody using a viral vector. For example, a full length heavy and light chain cDNA, including the cytoplasmic and transmembrane domains, is inserted into a linearized retrovirus vector. The plasmid encoding the retroviral vector and heavy and light chains is then transfected by calcium-phosphate precipitation into a packaging cell line. Supernatant from virus-producing cells are then filtered and tested for the presence of replication competent virus. The target cell line (for example a patient's lymphoblastoid cell line immortalized with the Epstein-Barr virus) expressing the correct MHC class II molecule is then transduced either by co-cultivation with the retrovirus packaging cell line or by retroviral supernatant infection, as is well known by those skilled in the art, e.g. by Moreau-Gaudry et al. in *Blood* (1995) 85:1449.

After generating suitable T cell clones, these are then used to map precisely the corresponding epitope. The corresponding amino-acid residues are then modified (i.e. mutated and/or deleted) in the full-length FVIII molecule by using methods well known in the art to identify the amino-acid residues which are critical for interaction with T cells. As a quality control measure, the resulting FVIII mutant molecules are then assessed for reactivity with a panel of T cell clones directed towards the region where the mutation/deletion has been introduced.

The production of antibodies towards soluble antigens requires that the antigen is first presented to the immune system in a manner suitable for recognition. Specialized cells, called antigen-presenting cells (hereinafter referred as APC) have the function to absorb the antigen, digest it by a combination of hydrolytic enzymes before associating the digested antigen fragments with molecules of the MHC class II complexes. The resulting complexes are then migrating to the surface of the cells for presentation to T cells. MHC class II complex molecules are constituted of a cleft that is open on both sides, allowing peptides of various lengths to bind. Such binding involves usually four or five major amino-acid residues that interact directly with corresponding residues in the MHC molecule via hydrophobic and electrostatic attractions. The anchoring residues are not contiguous but rather located two or three amino-acids apart. Examples of such anchoring residues can be found e.g. in Hammer et al., *J. Exp. Med.* (1994) 180:2353-8. Such anchoring residues can to some extent be predicted using available algorithms such as Tepitope (Raddrizzani et al., *Briefings in Informatics* (2000) 1:179-189), but can also be determined by *in vitro* experiments, using for instance competitive binding assays on soluble MHC molecules as disclosed for instance by Wall et al, *J. Immunol.* (1994) 152:4526.

The function of the anchoring residues is to maintain the T cell epitope in a fixed conformation, which is then recognizable by the antigen receptor of a T cell. The latter recognizes mostly a conformation made by both residues of the T cell epitope, but also by side residues of the MHC molecule. Some degree of flexibility exists in the actual amino-acid residues recognized by T cells: limited amino-acid

substitution can be carried out with no loss of T cell receptor binding, provided that the overall three-dimensional conformation is maintained.

The avidity of T cells for such peptide-MHC class II molecules complex is relatively low. This is compensated by the recognition of multiple peptides at the surface of the APC by T cell receptors. The interaction between APC and T cells is further reinforced by mutual recognition of a number of complementary surface molecules, such as CD40-CD40L, CD80/CD86 and CD28, the CD4 molecule, etc. Detailed description of these events can be found for instance in Lenschow et al., *Annu. Rev. Immunol.* (1996) 14:233-258 and in Oxenius et al., *Adv. Immunol.* (1998) 70:313-367. Following recognition of a peptide, the T cell undergoes a number of activation events starting by the phosphorylation of cytoplasmic proteins coupled to the T cell receptor. One of the consequences of such activation is the production of soluble mediators called interleukins, which participate in the tuning of B cell functions.

B cells, by contrast with T cells, recognize the antigen directly through their specific surface immunoglobulins. B cells are the main APC for secondary and subsequent immune responses, while professional APC (macrophages or dendritic cells) are the main APC for primary responses, when no specific B cells of sufficient avidity for the antigen are present. Upon re-exposure to the antigen, the latter is internalized by B cells via surface immunoglobulins, which deliver the antigen into the late endosomal compartment for processing and binding to MHC class II molecules, much alike professional APC. The cognate interactions between specific T cells and the B cell presenting the antigen is followed by activation of the T cell, with the aim of providing B cells with the signals necessary for full activation and maturation into antibody-producing cells. In the absence of such interaction between B and T cells, and therefore in the absence of specific T cells, no antibody production is elicited.

Hemophilia A is a hereditary disease characterized by the lack or insufficient function of FVIII. Patients suffering from such disease are usually treated by infusion of purified FVIII obtained by plasma purification or by genetically-

engineered mammalian cells transfected to produce FVIII. One major complication of FVIII infusion if the elicitation of a specific immune response towards infused FVIII. This immune response includes the production of high-affinity IgG antibodies, of which some inhibit the function of FVIII. The production of such inhibitor antibodies requires the presence of specific T cells, which provide B cells with the necessary signals to mature into antibody-secreting plasmocytes. The involvement of T lymphocytes in the development of antibodies against FVIII was shown by Singer et al. in *Thromb. Haemost.* (1996) 76:17-22 and by Reding et al. in *Thromb. Haemost.* (2000) 84: 643-52.

A more complete answer to the question of the presence of FVIII-specific T cells in haemophilia A patients is provided by the present invention. Thus, T cell clones have been derived from peripheral lymphocytes of a patient suffering from mild haemophilia and with an inhibitor antibody to wild-type FVIII, as described in more details in Example 1 below.

Antibodies to FVIII belong to the immunoglobulin-G (IgG) class, with only rare exceptions. Such IgG antibodies are of high affinity, suggesting that their production occurs only in a context of help provided by specific T cells. It is indeed well known by those skilled in the art that the production of antibodies with high affinity to soluble antigens is the result of a T cell driven maturation of the immune response in the presence of the antigen. A general overview of the mechanisms sustaining the production of antibodies can be found in Fanning et al., *Clin. Immunol. Immunopathol.* (1996) 79:1-14. Further evidence for T cell dependency is suggested by the observation that a high proportion of anti-FVIII antibodies belong to the IgG4 sub-type, the selection of which is known to be strictly T cell dependent.

Taken together, these data provide evidence that the production of anti-FVIII antibodies is dependent on the presence of FVIII-specific T cells and therefore that preventing or switching off specific T cell activation will with high probability result in prevention or suppression of the production of anti-FVIII antibodies.

Since FVIII is a molecule of high molecular weight, the number of possible T cell epitopes is large. This renders the identification of pathogenic epitopes, namely the epitopes that activate T cells participating with B cells in the production of inhibitory antibodies, rather elusive. This is well illustrated by the findings of Reding et al. (cited *supra*), who identified a large number of peptides scattered over the entire length of FVIII and able to activate T cells. The present inventors have now discovered a method suitable to identify relevant, pathogenic T cell epitopes, comprising deriving corresponding T cells from the blood of patients with haemophilia A developing an immune response towards exogenous FVIII. In the following examples, the effectiveness of the method of the invention is demonstrated by using cells from patients with mild/moderate forms of haemophilia A recognizing only exogenous FVIII, however the method can be applied to any patient developing anti-FVIII antibodies.

FVIII is a normal constituent of the coagulation pathway and as such belongs to self proteins. In order to prevent the emergence of an immune response against self-constituents and auto-immunity, nature has developed highly efficient means by which the immune system is depleted of cells (B and T lymphocytes) with reactivity towards self components. Thus, one of the functions of the thymus is to select and sort out the T cell repertoire so as to eliminate T cells reacting against self proteins. Detailed description of the different mechanisms at play in the thymus can be read e.g. in Plum et al., *Ann. NY Acad. Sci.* (2000) 917: 724-731.

T cell epitopes are organized according to a hierarchy including major, minor and cryptic epitopes. Cryptic epitopes are not processed and presented to T cells when the full-length protein is used. Such cryptic epitopes can however be detected when peptides from the protein are used instead. Under such conditions, peptides can be efficiently processed and therefore presented by APC to T cells. Because of lack of presentation in the thymus during the selection of the T cell repertoire, T cells recognizing cryptic epitopes are not eliminated and can be found in the periphery. Hence, peripheral blood T cells can be found which proliferate when presented with peptides of the corresponding proteins (see Reding et al.,

cited *supra*). Major and minor T cell epitopes are presented during thymus selection, which results in the elimination of corresponding T cells.

In mild/moderate hemophilia A, patients have significant levels of circulating FVIII. This FVIII is almost identical to wild-type FVIII, except for the region where the mutation/deletion is located. Thymus selection in such patients is therefore operating normally for all T cells reacting towards major and minor determinants, except for those determinants that are altered by the mutation/deletion itself. Such patients are therefore at risk of developing an immune response to FVIII when exposed to normal wild-type FVIII used for replacement therapy. The immune response is then directed only to wild-type FVIII and not towards the patient's self-FVIII.

The mutation/deletion occurring in the FVIII gene in mild/moderate hemophilia A patients can be easily identified by those skilled in the art. Methods to carry out such identification can be found in Higuchi, *Proc. Nat. Acad. Sci.* (1991) 88:8307-8311. When an immune response develops towards wild-type FVIII in mild/moderate hemophilia A patients, it should be primarily directed towards the region of FVIII containing the mutation/deletion. If the plasma contains anti-FVIII antibodies, the peripheral blood of such patients should contain FVIII-specific T cells whose property is to activate FVIII-specific B cells to produce anti-FVIII antibodies.

FVIII-specific T cells from mild/moderate hemophilia A patients having inhibitory antibodies towards wild-type FVIII therefore represent a unique source of material to select pathogenic T cells. Such T cells can be expanded and cloned by techniques well-known in the art, and which are described in more details in Example 1 below. T cell clones can be used to determine the precise epitope recognized and identify amino-acid residues involved in either MHC class II anchoring or T cell receptor recognition. This can be carried out using methods such as T cell activation with series of peptides with sequence varying by only one amino-acid at a time from the wild-type sequence. The basic principles of these methods are known in the art and are illustrated in Example 2 below. In addition,

residues binding to MHC class II molecules can be identified using purified MHC molecules of different haplotypes and inhibition of binding assays, as reported by Wall et al. (cited *supra*).

Amino-acid residues involved in the binding of the T cell epitope in MHC class II molecules are then mutated/deleted in the cDNA coding for FVIII. Mammalian cells such as Chinese Hamster Ovary (CHO) cells or Baby Hamster Kidney (BHK) cells are then transfected with mutated/deleted cDNA for production of a new mutated FVIII molecule. The latter is then tested for lack of activation of T cell clones and for the presence of co-factor activity in the coagulation cascade. Methods to introduce a mutation or delete DNA base pairs are well known in the art, as well as methods used to transfect mammalian cells and to produce recombinant FVIII molecules. Such methods can be found in general methodology reviews such as Current Protocols in Molecular Biology and Current Protocols in Protein Science (Wiley & Sons, Inc.). Example 2 below provides further details on such methods. The functional activity of the resulting modified FVIII molecule is then tested in either coagulation assays or chromogenic assays as described by Jacquemin et al. in *Blood* (2000) 96:958.

Reduced immunogenicity of the modified FVIII molecules of the present invention can be assessed in different systems. For example, an immunodeficient mouse strain can be used, the immune system of which is reconstituted with immunocompetent cells of human origin. Thus, severe combined immunodeficiency mice (hereinafter referred as SCID) can be reconstituted by intraperitoneal injection of peripheral blood lymphocytes of a haemophilia A patient presenting with an inhibitor. Such lymphocytes include immunocompetent B cells for antibody production and T cells to provide the necessary help to B cells in the production of anti-FVIII inhibitory antibodies. Series of mice can be reconstituted with cells of a single donor. Some of such mice are then immunized with wild-type FVIII, against which the patient is producing inhibitor antibodies, which results in the production of inhibitor antibodies. These can be detected by standard inhibition of coagulation assays well known in the art. Another group of mice is immunized

with the newly produced mutated FVIII molecule. In such case, it can be demonstrated whether a reduced response or no antibody response is elicited. Methods for the use of SCID mice are readily available, for instance from Vanzieleghem et al., *Thromb. Haemost.* (2000) 83:833-9.

The invention also provides a method by which T cell epitopes can be identified in other proteins of the coagulation cascade which are known to elicit immune responses altering the normal physiological activity of such protein. One particular example of such protein is FIX. Antibodies to FIX have been described in patients suffering from hemophilia B, which is due to a lack or insufficient function of FIX, as disclosed by Lusher in *Semin. Thromb. Haemost.* (2000) 26:179. The latter is an enzyme which activates factor X in the coagulation cascade. The invention therefore further provides a method, along the lines disclosed herein-above, to produce new recombinant FIX molecules for replacement therapy in patients suffering from hemophilia B.

The invention further provides a method to identify pathogenic T cell epitopes in other blood coagulation proteins such as factor X, factor V, factor VII, protein S and protein C. Antibodies inhibiting the function of such proteins have been described in Kunkel, *Hematol. Oncol. Clin. North Am.* (1992) 6:1341-1357.

As previously indicated, the recombinant human proteins and expression vectors of the invention are useful as therapeutically active ingredients for the manufacture of pharmaceutical compositions comprising the same and further comprising at least one pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" as used herein means any material or substance with which the active ingredient is formulated in order to facilitate its application or dissemination to the locus to be treated, for instance by dissolving, dispersing or diffusing the said composition, and/or to facilitate its storage, transport or handling without impairing its effectiveness. The pharmaceutically acceptable carrier may be a solid or a liquid or a gas which has been compressed to form a liquid, i.e. the compositions of this invention can suitably be used as

concentrates, emulsions, solutions, granulates, dusts, sprays, aerosols, suspensions, ointments, creams, tablets, pellets or powders.

Suitable pharmaceutical carriers for use in the said pharmaceutical compositions and their formulation are well known to those skilled in the art, and there is no particular restriction to their selection within the present invention. In particular, they include other human proteins, e.g. human serum albumin, as described for example in Remington's Pharmaceutical Sciences by E.W. Martin.

The pharmaceutical compositions of the invention may also include other additives such as wetting agents, dispersing agents, stickers, adhesives, emulsifying agents, solvents, coatings, antibacterial and antifungal agents (for example phenol, sorbic acid, chlorobutanol), isotonic agents (such as sugars or sodium chloride) and the like, provided the same are consistent with pharmaceutical practice, i.e. carriers and additives which do not create permanent damage to mammals, in particular to humans. The pharmaceutical compositions of the present invention may be prepared in any known manner, for instance by homogeneously mixing, coating and/or grinding the active ingredient, in a one-step or multi-steps procedure, with the selected carrier material and, where appropriate, the other additives such as surface-active agents. They may also be prepared by micronization, for instance in view to obtain them in the form of microspheres usually having a diameter of about 1 to 10 µm, namely for the manufacture of microcapsules for controlled or sustained release of the active ingredients.

Suitable surface-active agents to be used in the pharmaceutical compositions of the present invention are non-ionic, cationic and/or anionic materials having good emulsifying, dispersing and/or wetting properties. Suitable anionic surfactants include both water-soluble soaps and water-soluble synthetic surface-active agents. Suitable soaps are alkaline or alkaline-earth metal salts, unsubstituted or substituted ammonium salts of higher fatty acids (C_{10}-C_{22}), e.g. the sodium or potassium salts of oleic or stearic acid, or of natural fatty acid mixtures obtainable form coconut oil or tallow oil. Synthetic surfactants include sodium or calcium salts of polyacrylic acids; fatty sulphonates and sulphates;

sulphonated benzimidazole derivatives and alkylarylsulphonates. Fatty sulphonates or sulphates are usually in the form of alkaline or alkaline-earth metal salts, unsubstituted ammonium salts or ammonium salts substituted with an alkyl or acyl radical having from 8 to 22 carbon atoms, e.g. the sodium or calcium salt of lignosulphonic acid or dodecylsulphonic acid or a mixture of fatty alcohol sulphates obtained from natural fatty acids, alkaline or alkaline-earth metal salts of sulphuric or sulphonic acid esters (such as sodium lauryl sulphate) and sulphonic acids of fatty alcohol/ethylene oxide adducts. Suitable sulphonated benzimidazole derivatives preferably contain 8 to 22 carbon atoms. Examples of alkylarylsulphonates are the sodium, calcium or alcanolamine salts of dodecylbenzene sulphonic acid or dibutyl-naphtalenesulphonic acid or a naphtalene-sulphonic acid/formaldehyde condensation product. Also suitable are the corresponding phosphates, e.g. salts of phosphoric acid ester and an adduct of p-nonylphenol with ethylene and/or propylene oxide, or phospholipids. Suitable phospholipids for this purpose are the natural (originating from animal or plant cells) or synthetic phospholipids of the cephalin or lecithin type such as e.g. phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerine, lysolecithin, cardiolipin, dioctanylphosphatidyl-choline, dipalmitoylphoshatidyl -choline and their mixtures.

Suitable non-ionic surfactants include polyethoxylated and polypropoxylated derivatives of alkylphenols, fatty alcohols, fatty acids, aliphatic amines or amides containing at least 12 carbon atoms in the molecule, alkylarenesulphonates and dialkylsulphosuccinates, such as polyglycol ether derivatives of aliphatic and cycloaliphatic alcohols, saturated and unsaturated fatty acids and alkylphenols, said derivatives preferably containing 3 to 10 glycol ether groups and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon moiety and 6 to 18 carbon atoms in the alkyl moiety of the alkylphenol. Further suitable non-ionic surfactants are water-soluble adducts of polyethylene oxide with poylypropylene glycol, ethylenediaminopolypropylene glycol containing 1 to 10 carbon atoms in the alkyl chain, which adducts contain 20 to 250 ethyleneglycol ether groups and/or 10 to

100 propyleneglycol ether groups. Such compounds usually contain from 1 to 5 ethyleneglycol units per propyleneglycol unit. Representative examples of non-ionic surfactants are nonylphenol -polyethoxyethanol, castor oil polyglycolic ethers, polypropylene/polyethylene oxide adducts, tributylphenoxypolyethoxyethanol, polyethyleneglycol and octylphenoxypolyethoxyethanol. Fatty acid esters of polyethylene sorbitan (such as polyoxyethylene sorbitan trioleate), glycerol, sorbitan, sucrose and pentaerythritol are also suitable non-ionic surfactants.

Suitable cationic surfactants include quaternary ammonium salts, preferably halides, having 4 hydrocarbon radicals optionally substituted with halo, phenyl, substituted phenyl or hydroxy; for instance quaternary ammonium salts containing as N-substituent at least one C_8-C_{22} alkyl radical (e.g. cetyl, lauryl, palmityl, myristyl, oleyl and the like) and, as further substituents, unsubstituted or halogenated lower alkyl, benzyl and/or hydroxy-lower alkyl radicals.

A more detailed description of surface-active agents suitable for this purpose may be found for instance in "McCutcheon's Detergents and Emulsifiers Annual" (MC Publishing Corp., Ridgewood, New Jersey, 1981), "Tensid-Taschenbuch", 2nd ed. (Hanser Verlag, Vienna, 1981) and "Encyclopaedia of Surfactants (Chemical Publishing Co., New York, 1981).

Pharmaceutical forms suitable for injectionable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation thereof. Typical carriers for this purpose therefore include biocompatible aqueous buffers, ethanol, glycerol, propylene glycol, polyethylene glycol and the like, and mixtures thereof.

The present invention also provides the use of a human recombinant protein as a medicament. When the said protein is a blood coagulation factor, it is preferably used for preventing and/or treating disorders of hemostasis, in particular, coagulation disorders and other thrombotic pathologic conditions in mammals, preferably in humans. When the said protein is not a blood coagulation factor, but a protein involved in another biological process such as disclosed herein-above, it may be used for the treatment or prevention of diseases, a non-exhaustive list of

which was given herein-before in connection with examples of relevant proteins. The said recombinant protein may be provided to a patient by any means well known in the art, i.e. orally, intranasally, subcutaneously, intramuscularly, intradermally, intravenously, intraarterially, parenterally or by catheterization.

As an example, when human FVIII is the protein involved, the recombinant protein of the invention will usually be administered intravenously, in an amount of 40 units (1 unit FVIII being defined as the amount of FVIII present in 1 ml of a pool of plasma of normal individuals) per kg body weight of the patient for pre-operative indications, 15 to 20 units per kg body weight for minor bleeding episodes, and 20 to 40 units per kg body weight every day for maintenance dose or prophylaxis. For the treatment of patients who develop inhibitors to FVIII, doses up to 200 units per kg body weight may be administered twice a day.

The following examples are provided for the purpose of illustrating the FVIII embodiment of the present invention and should in no way be understood as limiting the scope of this invention which, as previously indicated, is widely applicable to a broad range of proteins, including blood coagulation factors.

EXAMPLE 1 - Determination of the T cell epitope recognised by FVIII-specific T cell clones.

A) expansion of FVIII-specific oligoclonal T cell lines

Peripheral blood mononuclear cells (PBMC) are purified by Lymphoprep density gradient centrifugation (available from Nycomed Pharma). In order to generate autologous dendritic cells, PBMC are depleted from T lymphocytes using CD4 and CD8 MicroBeads (available from Miltenyi Biotech), following the manufacturer's recommendations. The negatively selected cells were incubated for two hours at 37°C in culture flasks (Falcon) at a density of 2 x 10^6 cells/ml in Roswell Park Memorial Institute (hereinafter referred as RPMI) 1640 medium supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine and 1% autologous plasma previously heated at 56°C for 30 minutes, according to methods well known by those skilled in the art (e.g. Chaux et al. in *J. Exp. Med.* (1999) 189:767). Non-adherent cells are frozen in liquid nitrogen and used as a

source of B lymphocytes. Adherent cells are cultured in the presence of interleukin-4 (hereinafter IL-4) (100 U/ml) and granulocyte macrophage-colony stimulating factor (hereinafter referred as GM-CSF) (100 ng/ml) in RPMI-1% autologous serum. Half of the medium was replaced on day 2 and day 4 with fresh medium plus interleukins.

On day 5, autologous dendritic cells were incubated at 37°C, 5% CO_2 for 18 hours in RPMI-1% autologous serum supplemented with IL-4 (100 u/ml), GM-CSF (100 ng/ml), and tumor necrosis factor-alpha (hereinafter TNF-α) (1 ng/ml) in the presence of 20 µg/ml plasma-derived or recombinant FVIII. Cells were washed and added at 10^4 per well to 10^5 $CD4^+$ lymphocytes (isolated using CD4 MicroBeads as above) in 200 µl Iscove's modified Dulbecco's medium (hereinafter IMDM) (available from Gibco BRL) supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine and 10% human serum in the presence of IL-6 (1.000 U/ml) and IL-12 (10 ng/ml). The CD4+ lymphocytes were re-stimulated on day 7, day 14 and day 21 with autologous dendritic cells freshly loaded with FVIII and were grown in IMDM-10% human serum supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). The cells containing proliferating $CD4^+$ T cells were assessed on day 35 for the presence of FVIII-specific T cells.

B) detection of FVIII-specific oligoclonal T cells by IFN-γ production

Autologous Epstein-Barr virus immortalized B cells were incubated for 18 hours in the presence of 20 µg/ml of plasma-derived FVIII. FVIII-pulsed lymphoblastoid cells were washed and incubated at 5,000 per well in round bottomed microculture plates with 5,000 $CD4^+$ T cells in IMDM-10% human serum supplemented with IL-2 (25 U/ml). After 20 hours, the supernatants were collected and interferon-gamma (hereinafter IFN-γ) in the supernatant was measured by an ELISA assay using reagents from Medgenix Diagnostics Biosource.

C) isolation of CD4+ T cell clones

The cell lines that recognized cells loaded with FVIII were cloned by limiting dilution, using as stimulating cells FVIII-specific autologous lymphoblastoid cells

(from the cell line KRIX 1 deposited with the Belgian Coordinated Collections of Micro-organisms under accession number LMBP 5089CB) loaded with FVIII by incubation for 18 hours in the presence of 20 µg/ml FVIII. Allogeneic cells were added as feeders cells. Established clonal $CD4^+$ T cell lines were then grown in complete IMDM-10% human serum supplemented with IL-2 (50 U/ml), IL-7 (5 ng/ml) and 0.5 µg/ml phytohemagglutinin (HA 16, available from Murex Diagnostics) according to well known methods (e.g. Chaux et al., cited *supra*). Using these methods, two T cell clones, named hereinafter B3:6 and D9:E9, were isolated.

D) characterization of T cell clones

The procedure of paragraph 1-B above was repeated. As shown in Figure 1, when three T cell clones are incubated with autologous lymphoblastoid cell lines expressing FVIII-specific surface immunoglobulin (KRIX 1) and loaded with FVIII, high amounts of IFN-γ (1,200-1,600 pg/ml) are detected in culture supernatant. This response is specific for FVIII as indicated by the 10-times lower production of IFN-γ in the absence of FVIII. Similar results were obtained with recombinant FVIII, indicating that T cells were specific for FVIII and not for other contaminant proteins present in FVIII concentrates purified from plasma.

In order to determine the location of the T cell epitopes in the FVIII molecule, T cells are incubated with recombinant fragments corresponding to different domains of the FVIII molecule and produced in *E.Coli* according to Jacquemin et al. in *Blood* (1998) 92:496-506. As shown in Figure 2, only the native C1 domain induces secretion of IFN-γ by the two T cell clones. No IFN-γ secretion is induced using the C2 domain (Figure 2) or other recombinant fragments corresponding to the A1, A2 or A3 domains (data not shown). The C1 domain carrying substitution Arg2150 -> His does not stimulate IFN-γ production by B3:6. However IFN-γ is produced following incubation with D9 :E9, although IFN-γ concentration remains lower than with the native C1 domain. These data demonstrate that substitution of Arg2150 by His eliminates a T cell epitope recognized by B3:6 but only partially alters the epitope recognized by the T cell

clone D9:E9. These data suggest that the T cell epitopes recognized by the two T cell clones are located in the C1 domain and that they include amino-acid residue 2150.

E) mapping of the T cell epitopes using T cell clones and synthetic peptides

The T cell epitope is further defined using synthetic peptides encompassing residue 2150. As shown in Figure 3, a peptide encompassing residues 2144-2161 stimulates the T cell clones B3:6 and D9:E9. The epitope specificity of the T cell clones is further defined using shorter synthetic peptides. A peptide encompassing residues 2148-2161 stimulates B3:6, but not D9:E9, indicating that the specificity of the latter is different. The synthetic peptide encompassing residues 2144-2158 also stimulates both T cell clones, as shown in Figure 3.

F) Comparison of FVIII-specific and non FVIII-specific lymphoblastoid cell lines as antigen-presenting cells

FVIII-specific B cell lines are used as antigen-presenting cells during cloning of FVIII-specific T cells. This significantly differs from previously published methods (e.g. Chaux et al. cited *supra*) known to generate specific T cell clones directed toward proteins other than FVIII, in which non-specific autologous antigen-presenting cells were used. Prior art indeed indicates that an antibody can alter antigen processing, particularly when the antibody is directed towards a region that also contains a T cell epitope. Synthetic peptides were efficiently presented by FVIII-specific and non-specific lymphoblastoic cell lines, as shown by figure 4, which indicated that these cells expressed the correct MHC class II molecules. These unexpected results indicated that FVIII-specific cell lines should preferably be used to generate and clone FVIII-specific T cells when the epitope of the T cell clone is unknown and complete FVIII molecule must be used an antigen.

EXAMPLE 2 - identification of substitutions altering T cell epitopes

The activation of the T cell clones by the wild type FVIII peptide 2144-2161 was compared with activation in the presence of the synthetic peptide 2144-2161 carrying the substitution of arginine by histidine at residue 2150. No production of IFN-γ was detected when the T cell clone B3:6 was stimulated with the said

synthetic peptide. The T cell clone D9:E9 produced a significant amount of IFN-γ following stimulation with the mutated peptide, although the response was reduced by comparison to that observed with the wild type peptide, as shown in figure 5. The fact that substitution Arg2150His completely prevents activation of T cell clone B3:6 but only partially that of clone D9:E9 is in agreement with the observation that these two T cell clones recognize different epitopes, as already shown in figure 3. It also indicates that a FVIII molecule carrying this substitution will have a significant, but only partial, antigenicity for haemophilia A patients T cells. This observation is also in agreement with the observation that several patients carrying the substitution Arg2150His who developed an immune response towards exogeneous FVIII also develop an immune response towards their own FVIII according to Santagostino et al., *Thromb. Haemost.* (1995) 74:619.

Similarly, the analysis of other synthetic peptides carrying point mutations indicates that substitution Thr2154Ile prevents recognition by B3:6 but not by D9:E9 (as shown in figure 5), also in agreement with the observation that these two T cell clones recognize different epitopes. Unexpectedly, the substitution Pro2153Gln completely prevents recognition by both T cell clones (as shown in figure 5). Similar results are obtained using a recombinant FVIII molecule carrying the substitution Pro2153Gln.

Interestingly, analysis of the activity of a FVIII molecule carrying the substitution Pro2153Gln indicates that its pro-coagulant activity is close to normal, as shown below.

These observations indicate that FVIII molecules carrying the substitution Pro2153Gln have a completely abrogated antigenicity with regards to the T cell clones B3:6 and D9:E9.

Given the T-cell dependency of the development of humoral response towards FVIII, the administration of such a mutated FVIII molecule to haemophilia A patients will result in a reduced propensity to develop such a humoral response.

EXAMPLE 3 - production and characterization of recombinant mutated FVIII.

A) plasmid mutagenesis

Mutagenesis is performed within a mammalian expression vector coding for the FVIII cDNA, according to Vehar et al. in *Nature* (1984) 312:337. Also suitable for this purpose is the vector encoding the B domain-less FVIII cDNA according to Lind et al., *Eur. J. Biochem.* (1995) 232:19-27. Mutant plasmids are generated through oligonucleotide site-directed mutagenesis utilizing the polymerase chain reaction in accordance with Jacquemin et al. in *Blood* (2000) 92:496. Codon No. 2150 was mutated from CGT to CAT, predicting an arginine to histidine amino-acid change. Codon No. 2153 was mutated from CCA to CAA, predicting an amino acid change from Pro to Gln. Codon No. 2201 was deleted. All mutated cDNAs are controlled by sequencing in both directions using a Genetic Analyzer 3.10 from Perkin Elmer.

B) Chinese hamster ovary (CHO) cell transfection

In order to establish cell lines expressing wild-type or mutant FVIII, CHO cells are transfected with plasmid encoding wild-type or mutated FVIII using FUGENE 6 (Boehringer Mannheim, Brussels, Belgium) according to the manufacturer's instructions.

C) FVIII production by transfected CHO cells

Briefly, CHO cells (8 x 10^4 cells/well) were seeded in 6-well plates (Life Technologies) using minimal essential medium alpha (hereinafter MEM-α, available from Life Technologies Ltd., Paisley, United Kingdom) supplemented with 10% fetal calf serum (hereinafter FCS). After 24 hours incubation, a transfection mixture of 0.5 µg DNA in 10 µl of tris-ethylenediamine tetraacetic acid (EDTA), 100 µl OPTIMEM and 2 µl FUGENE® 6 (available from Roche) was applied to the cells. After 48 hours, the cells were washed twice with MEM-α and the culture medium was replaced by MEM-α supplemented with Nutridoma-CS® (Boehringer Mannheim, Germany) and 3 mM sodium butyrate. After 16 hours, the conditioned medium was harvested, centrifuged to remove cell debris and assayed for FVIII

activity. The cell lines producing the highest FVIII amounts are expanded and sub-cloned twice.

D) evaluation of the specific activity of the FVIII molecule

The cofactor activity of recombinant FVIII molecules is evaluated using the FVIII chromogenic assay available from Dade AG (Switzerland) according to the manufacturer's recommendations. In this assay, thrombin-activated FVIII accelerates the conversion of factor X into factor Xa in the presence of factor IXa, phosppholipids (hereinafter PL) and calcium ions; factor Xa activity is then assessed by hydrolysis of a p-nitroanilide substrate. Reagents, which were reconstituted according to the manufacturer's instruction, comprised bovine factor X (1 mM), factor IXa (0.3 mM) and thrombin (0.3 mM); $CaCl_2$ (30 mM), PL (60 mM), a chromogenic factor Xa substrate (CH3OCO-D-CHG-gly-Arg-pNA.AcOH; 3.4 mM) and a thrombin inhibitor (L-amidinophenylalanine piperidine) according to Jacquemin et al. in *Blood* (1998) 92:496.

Plasma FVIII antigen levels were measured in ELISA using the Immunozym FVIII:Ag® test (available from Immuno AG, Vienna, Austria) following the manufacturer's recommendations. Recombinant FVIII antigen levels were also measured in ELISA according to published methods, using monoclonal antibodies F4H12 or F15B12 recognizing the A1 or A2 domain of FVIII respectively, as capture antibodies. Bound FVIII was detected by the addition of a mixture of monoclonal antibodies 13, F8D6, F29A1 and F14A12, according to Jacquemin et al., *Blood* (2000) 96:962.

The FVIII specific activity is calculated as indicated before under the heading "Definitions". Dysfunctional FVIII molecules with specific activity as low as 0.01 were reported for FVIII variants, also called cross-reactive material positive FVIII in order to emphasize the disparity between the amount of FVIII protein and the level of FVIII functional activity, by Pemberton et al. in *Blood* (1997) 89:2413. Interestingly, the substitution Pro2153Gln results in a FVIII molecule with a specific activity close to normal. Similarly, we demonstrated that deletion of a single amino-acid residue, such as amino-acid 2201 in the FVIII C2 domain resulted in a FVIII

molecule with a specific activity close to 1. This result is quite unexpected because this residue is located close to residues 2199 and 2200 which are known to mediate FVIII binding to phospholipids, an essential feature of FVIII, according to Pratt et al. in *Nature* (2000) 402:439-42.

Deletion of a single amino-acid residue, even in a region important for FVIII functional activity such as the C2 domain where this residue is located, can therefore be considered as a suitable alternative to the substitution of a defined residue. The surprising observations made with the Pro2153Gln substituted FVIII molecule indicate that it is possible to produce recombinant FVIII molecules which are not recognized by all T cell clones available so far while preserving a FVIII activity sufficient to guarantee haemostasis. Accordingly, these FVIII molecules are less immunogenic because T cell clones recognizing this epitope will not be activated following administration.

CLAIMS

1. A recombinant mammalian protein modified to eliminate or reduce by at least about 80% the activation of at least one T-cell clone derived from a mammal with antibody against the wild-type protein, with respect to activation by the wild-type protein, the said recombinant protein having a specific activity higher than 0.1.

2. A recombinant mammalian protein according to claim 1, wherein the T cell clone is derived from a human.

3. A recombinant protein according to claim 1 or claim 2, wherein the said protein is a coagulation factor.

4. A recombinant protein according to any of claims 1 to 3, wherein the said protein is factor VIII.

5. A recombinant protein according to any of claims 1 to 3, wherein the said protein is factor IX.

6. A recombinant protein according to any of claims 1 to 5, being in a purified form.

7. A method to generate a mammalian protein-specific T-cell clonal cell line by using a cell line expressing protein-specific antibodies, or fragments thereof, on its surface.

8. A method according to claim 7, wherein the cell line is a lymphoblastoid cell line.

9. A method according to claim 7 or claim 8, wherein the said protein is a coagulation factor.

10. A method according to claim 9, wherein the said coagulation factor is factor VIII.

11. A method according to claim 9, wherein the said coagulation factor is factor IX.

12. A method according to any of claims 7 to 11, wherein cell lines expressing the protein-specific antibodies are obtained by transfection or transduction with an expression vector for the protein-specific antibody or fragment thereof.

13. A method according to any of claims 7 to 12, comprising the step of identifying a peptide which has an epitope recognized by the clonal T-cell line by using a synthetic peptide library corresponding to the said protein.

14. A method according to claim 13, further comprising the step of using the T cell clonal cell line to identify modifications of the T-cell epitope eliminating or reducing by at least about 80% its ability to activate the T-cell clonal cell line.

15. A method according to claim 14, further comprising the step of producing a recombinant protein carrying a modification identified in the previous identification step.

16. A method according to claim 15, further comprising the step of using the T-cell clone to verify that the modified protein does not provoke more than 20% T-cell activation by comparison to the wild-type protein.

17. A method according to claim 16, further comprising the step of controlling the activity of the modified protein by means of a suitable protein functional assay.

18. A recombinant protein according to any of claims 1 to 6, being obtainable by a method according to any of claims 7 to 17.

19. A recombinant protein according to any of claims 1 to 6 and 18, carrying, in respect of the wild-type protein, a substitution of a single residue and/or one amino-acid deletion.

20. A recombinant protein according to claim 19, wherein the protein is human factor VIII and wherein the said recombinant protein carries a substitution by another residue in the region between residues 2144 and 2161.

21. A recombinant human protein according to claim 20, wherein the substitution is at residue 2153.

22. A recombinant protein according to claim 19, wherein the protein is human factor VIII and wherein the said recombinant protein carries a deletion of residue 2201.

23. A recombinant protein according to any of claims 1 to 5 and 18, carrying in respect of the wild-type protein several modifications located in one domain or combination of domains of the said protein.

24. A peptide identified by using a T-cell clonal cell line generated by a method according to claim 13.

25. A peptide according to claim 24, encompassing residues which can be mutated or deleted to eliminate or reduce by at least about 80% the activation of at least one T-cell clone activated by a wild-type protein.

26. A peptide according to claim 24 or claim 25, wherein the protein is a coagulation factor.

27. A peptide according to claim 26, wherein the coagulation factor is human factor VIII and wherein the said peptide includes at least residues 2144 to 2161 thereof.

28. A peptide according to claim 26, wherein the coagulation factor is human factor IX.

29. In vitro use of a peptide according to any of claims 24 to 28 for evaluating T-cell reactivity.

30. In vivo use of a peptide according to any of claims 24 to 28 for evaluating and/or modulating T-cell reactivity.

31. A DNA sequence coding for a recombinant protein according to any of claims 1 to 6 and 18 to 23.

32. A DNA sequence according to claim 31, being the DNA sequence of human factor VIII carrying one single mutation at residue 2153.

33. A DNA sequence according to claim 31, being the DNA sequence of human factor VIII carrying a deletion of residue 2201.

34. An expression vector including a DNA sequence according to any of claims 31 to 33 and a suitable promoter.

35. A pharmaceutical composition comprising an effective amount of a recombinant protein according to any of claims 1 to 6 and 18 to 23 or a peptide according to

any of claims 24 to 28 or an expression vector according to claim 34, and a pharmaceutically acceptable carrier.

36. A method of prevention or treatment of a disease induced by a lack or a dysfunction of a human protein, comprising administering to a human patient in need thereof an effective amount of a recombinant protein according to any of claims 1 to 6 and 18 to 23 or a peptide according to any of claims 24 to 28 or an expression vector according to claim 34.

37. A method of prevention or treatment according to claim 36, wherein the said protein is a coagulation factor.

38. A method of prevention or treatment according to claim 37, wherein the said coagulation factor is factor VIII and the said disease is haemophilia A.

39. A method of prevention or treatment according to claim 37, wherein the said coagulation factor is factor IX and the said disease is haemophilia B.

40. A T-cell clonal cell line generated by a method according to any of claims 7 to 17.

41. A T-cell clonal cell line according to claim 40, being obtained by using as antigen presenting cell the cell line KRIX 1 deposited with the Belgian Coordinated Collections of Micro-organisms under accession number LMBP 5089CB.

42. A method to produce a recombinant mammalian protein with reduced immunogeneicity, comprising the steps of :

(a) isolating from a mammal antibody-producing T-cells against a wild-type mammalian protein,

(b) identifying the major T-cell epitopes of the said wild-type mammalian protein which are associated with activation of T cells obtained in step (a), and

(c) substituting or deleting amino-acids of the major T-cell epitopes identified in step (b) in such way as to eliminate or reduce by at least about 80% T cell activation.

43. A method according to claim 42, further comprising selecting the amino-acid substitutions or deletions of step (c) which are able to preserve a specific activity of the recombinant mammalian protein higher than about 0.1.

44. A method according to claim 42 or claim 43, wherein the identifying step (b) is performed by means of a peptide library from the wild-type mammalian protein.

45. A method according to any of claims 42 to 44, wherein the mammalian protein is a human protein.

46. A method according to any of claims 42 to 45, wherein the mammalian protein is a coagulation factor.

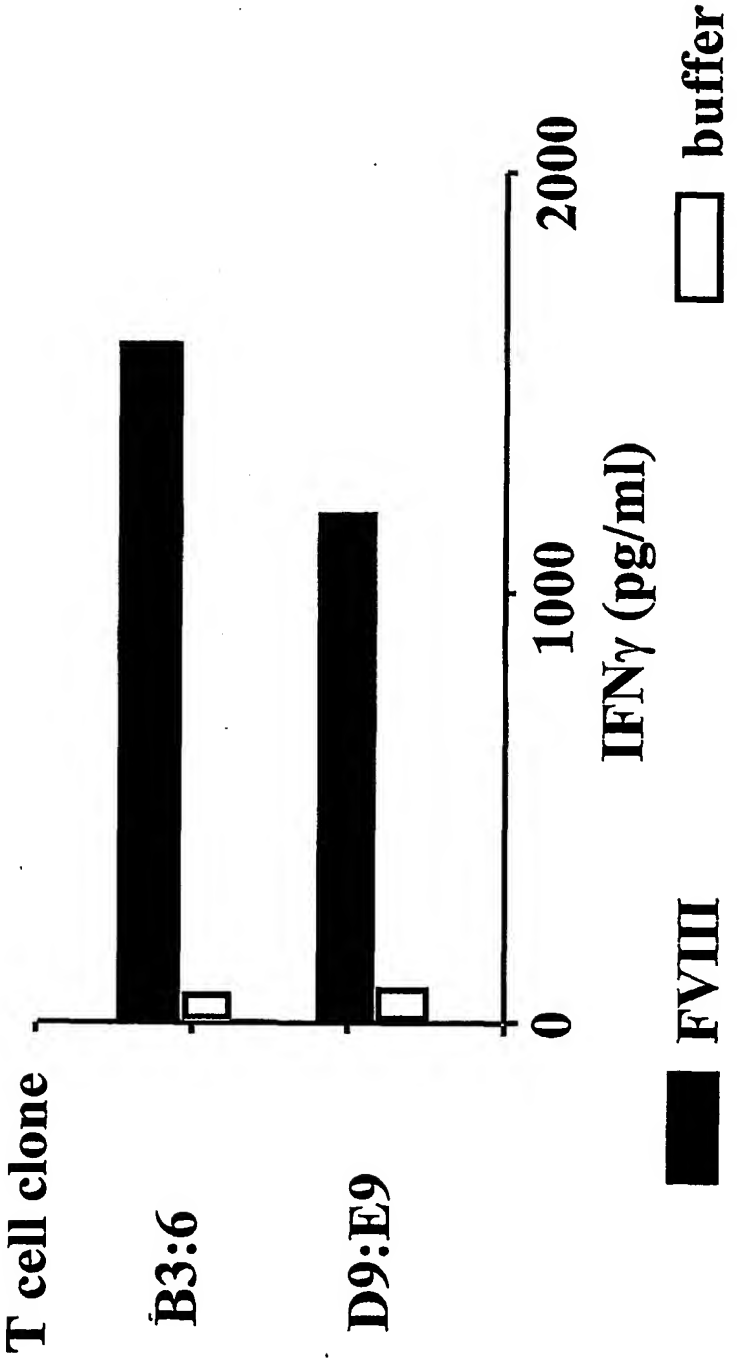

Figure 1

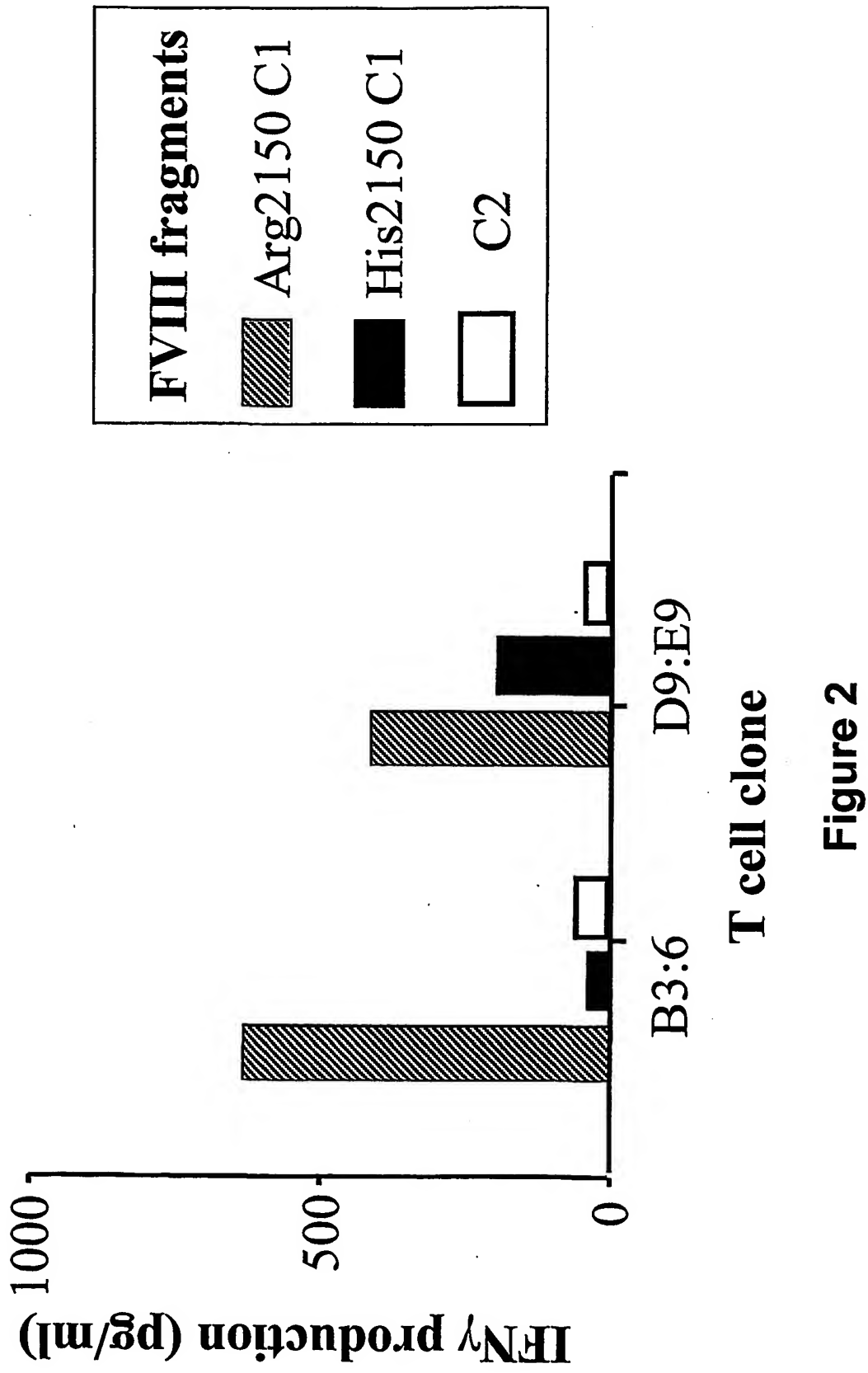
FVIII fragments
Arg2150 C1
His2150 C1
C2
IFNγ production (pg/ml)
1000
500
0
B3:6
D9:E9
T cell clone

Figure 2

Amino acid residues	T cell clone	
2140 2150 2160	B3:6	D9:E9
IFNPPIIARYIRLHPT	-	-
IIARYIRLHPTHYSIRST	++	++
ARYIRLHPTHYSIRST	+	+
YIRLHPTHYSIRST	+	-
RLHPTHYSIRST	-	-
IIARYIRLHPTHYSI	+	+
IIARYIRLHPTHY	-	-

Figure 3

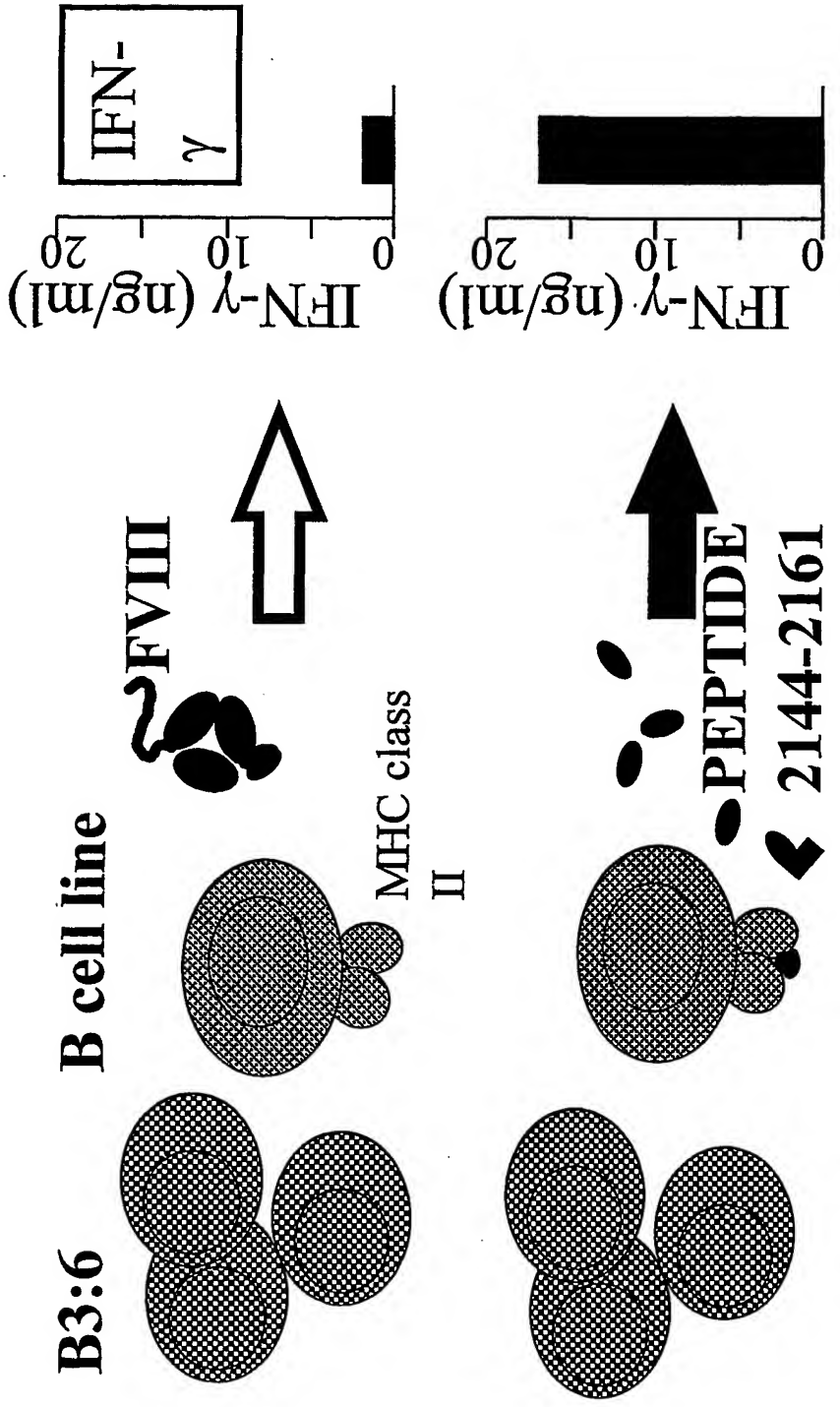
B3:6
B cell line
FVIII
MHC class II
PEPTIDE 2144-2161
IFN-γ (ng/ml)
0
10
20
IFN-γ
IFN-γ (ng/ml)
0
10
20

Figure 4

Amino acid residues	T cell clone		Mutation
2145 2160	B3:6	D9:E9	
IIAQYIRLHPTHYSIRST	++	++	Arg2147Gln
IIARYIHLHPTHYSIRST	-	+	Arg2150His
IIARYIRLHQTHYSIRST	-	-	Pro2153Gln
IIARYIRLHPIHYSIRST	+	-	Thr2154Ile
IIARYIRLHPTHYSILST	++	++	Arg2159Leu

Figure 5

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/37 C07K14/755

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, EMBASE, CHEM ABS Data, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAKAHASHI ISAO ET AL: "Change of antigenic and neutralizing specificity in substitutional epitope peptides of hemophilia B inhibitor." PEPTIDES (NEW YORK), vol. 19, no. 7, 1998, pages 1129-1136, XP001037525 ISSN: 0196-9781 the whole document --- -/--	1-3,5-9, 11-19, 23-26, 28-31, 34-37, 39-46

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 November 2001	12.04.02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Novak, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JACQUEMIN MARC ET AL: "A novel cause of mild/moderate hemophilia A: Mutations scattered in the factor VIII C1 domain reduce factor VIII binding to von Willebrand factor." BLOOD, vol. 96, no. 3, 1 August 2000 (2000-08-01), pages 958-965, XP002176173 ISSN: 0006-4971 abstract; figure 1; tables 1-3	1-4, 6-10, 12-23, 27, 29-38, 40-46
X	TAKAHASHI ISAO ET AL: "Epitope mapping of human factor IX inhibitor antibodies." BRITISH JOURNAL OF HAEMATOLOGY, vol. 88, no. 1, 1994, pages 166-173, XP001022869 ISSN: 0007-1048 the whole document	1-3,5-9, 11-19, 23-26, 28-31, 34-37, 39-46
X	JACQUEMIN M ET AL: "A human antibody directed to the factor VIII C1 domain inhibits factor VIII cofactor activity and binding to von Willebrand factor" BLOOD, W.B.SAUNDERS COMPAGNY, ORLANDO, FL,. US, vol. 95, no. 1, 1 January 2000 (2000-01-01), pages 156-163, XP002150704 ISSN: 0006-4971	1-4,6, 18-20, 27,31,34
Y	abstract; figure 4; table 1	7-10, 12-17, 21-23, 29,30, 32,33, 35-38, 40-46
Y	PEERLINCK KATHELIJNE ET AL: "Antifactor VIII antibody inhibiting allogeneic but not autologous factor VIII in patients with mild hemophilia A." BLOOD, vol. 93, no. 7, 1 April 1999 (1999-04-01), pages 2267-2273, XP002176174 ISSN: 0006-4971 the whole document	1-4, 6-10, 12-23, 27, 29-38, 40-46
Y	EP 0 426 913 A (BAXTER INT ;INT INST CELLULAR MOLECUL PATH (BE)) 15 May 1991 (1991-05-15) abstract; claims 1-9	1-4, 6-10, 12-23, 27, 29-38, 40-46

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	HIGH KA: "Factor IX: molecular structure, epitopes, and mutations asociated with inhibitor formation" ADV. EXP. MED. BIOL., vol. 386, 1995, pages 79-86, XP001037402 the whole document	1-3,5-9, 11-19, 23-26, 28-31, 34-37, 39-46
Y	WO 00 74688 A (PHILADELPHIA CHILDREN HOSPITAL) 14 December 2000 (2000-12-14) the whole document	1-3,5-9, 11-19, 23-26, 28-31, 34-37, 39-46
A	WO 99 53038 A (GENENCOR INT) 21 October 1999 (1999-10-21)	
A	JACQUEMIN M G ET AL: "MECHANISM AND KINETICS OF FACTOR VIII INACTIVATION: STUDY WITH AN IGG4 MONOCLONAL ANTIBODY DERIVED FROM A HEMOPHILIA A PATIENT WITH INHIBITOR" BLOOD, W.B. SAUNDERS, PHILADELPHIA, VA, US, vol. 92, no. 2, 1998, pages 496-506, XP000906844 ISSN: 0006-4971 abstract	
A	HEDNER U: "TREATMENT OF PATIENTS WITH FACTOR VIII AND FACTOR IX INHIBITORS WITH SPECIAL FOCUS ON THE USE OF RECOMBINANT FACTOR VIIA" THROMBOSIS AND HAEMOSTASIS, STUTTGART, DE, vol. 82, no. 2, August 1999 (1999-08), pages 531-539, XP001021613 ISSN: 0340-6245	
E	WO 01 40281 A (MAEYER MARC CYRIEL HILDA DE ;THROMB X N V (BE); PLAISANCE STEPHANE) 7 June 2001 (2001-06-07) the whole document	1-46

INTERNATIONAL SEARCH REPORT

ɜrnational application No.
PCT/EP 01/06297

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 24,25,26
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3, 6-9,12-19,23,29-31,34-37,40-46 (partially) ;4, 10,20-22,27,32-33,38 (completely)

   Recombinant mammalian proteins modified to eliminate or reduce by at least 80% the activation of at least one T-cell clone, said molecule represented by factor VIII, respectively methods to generate such proteins, and the uses thereof.

2. Claims: 1-3, 6-9,12-19,23-26,29-31,34-37, 40-46 (partially); 5,11,28,39 (completely)

   Recombinant mammalian proteins modified to eliminate or reduce by at least 80% the activation of at least one T-cell clone, said molecule represented by factor IX, respectively methods to generate such proteins, and the uses thereof.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 24,25,26

Present claims 24,25 and 26 relate to a protein defined by reference to a desirable characteristic or property, namely in that it is identified by using a T-cell clonal line.

The claims cover all proteins having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such proteins.
In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT).
An attempt is made to define the proteins by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.
Consequently, the search has been carried out for those parts which appear to be clear, supported and disclosed, namely those parts relating to the proteins wherein they are coagulation factors. VIII or IX.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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		DE 68926239 T2	05-12-1996
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		CN 1297484 T	30-05-2001
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		NO 20005153 A	11-12-2000
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		WO 9953038 A2	21-10-1999
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		WO 0140281 A2	07-06-2001